COLLEGE OF
Public Health
Division of Environmental Health Sciences

Final Report

Rapid Detection of
Bacteroides fragilis in Lake Erie

Submitted by:
Jiyoung Lee, PhD
375 Howlett Hall, 2001 Fyffe Ct.
Columbus, OH 43210
jlee@cph.osu.edu

Abstract

The beach water of Lake Erie continues to be contaminated by multiple sources. Beach waters are currently monitored using culture-based methods to protect swimmers from microbial hazards; however, such methods are not rapid and unable to discern sources of fecal contamination. In an effort to better understand these sources of fecal contamination, we quantified the human-associated fecal bacteria, *Bacteroidales*. This was accomplished using three recently developed genetic markers and a novel immunomagnetic separation (IMS) method targeting *Bacteroides fragilis*. The traditional 24-hour membrane filtration approach for *E. coli* and enterococci was also employed. The IMS method used in this study was able to generate a result in less than 2 hours upon sample collection. The genetic marker methods employed in the lab produced reliable results of human contamination within 3 hours of analyzing the sample. These results were correlated with *E.* coli and enterococci culture methods. The results regarding these 30 samples, 15 collected from Euclid and Villa Angela Beaches in Cleveland, demonstrated significant human-associated fecal contamination in nearly all samples. The method development this project funded enabled the acquisition of additional funding from USEPA for further clarification of the extent of human and waterfowl fecal contamination at these beaches.

Introduction

Euclid and Villa Angela Beaches are the two most impaired beaches in Ohio and are consistently among the most impaired in the U.S. among coastal beaches (Dorfman and Rosselot 2009). Additionally, the two beaches are within the Cuyahoga River Area of Concern, which is in need of significant improvements in recreational water quality as determined by this area's Remedial Action Plan (Ohio EPA 2008). This particular project increases the knowledge of the extent of human sources of pollution impacting the two study beaches at Villa Angela and Euclid.

Villa Angela and Euclid Beaches are among the few publically available beaches in the large metropolitan area of Cleveland, Ohio. With Euclid and Villa Angela experiencing advisory conditions 47% and 45% of the time, respectively, it is obvious remediation efforts are needed. Despite substantial infrastructure upgrades in the Cleveland community, fecal contamination is still problematic. With Ohio State Parks struggling to take remediation steps due to unfamiliarity with the source of the problem, action steps are warranted. Ohio's action plan calls for Ohio to have "safe, healthy, accessible coastal areas for all Ohioans." To get towards that goal, the 2009-2011 action steps call for the "development and implementation of technologies and field studies to identify and trace sources of local bacterial contamination.

Recently, with the advent of rapid molecular techniques, there has been numerous types of alternative indicators that previously could not be practically measured. With the new technology, the U.S. EPA has considered a number of alternative fecal indicator organisms for recreational waters (U. S. Environmental Protection Agency 2007). Among the suggested potential candidates, the *Bacteroides* genus has gained substantial interest, particularly

because of the relative abundance of certain genera in human feces and human-impacted waters (Holdeman *et al.* 1976; Layton *et al.* 2006; Converse *et al.* 2009).

For Ohio's Lake Erie Beaches, *E. coli* has traditionally been used as an indicator microorganism for communicating waterborne disease risk particularly because of their ability to be easily and safely cultured (Simpson *et al.* 2002) and is a standard method (EPA method 1603). However, most of the bacterial species composing the mammal gut microbiota are anaerobes that are not easy to enumerate by conventional culturing techniques and the majority of that flora is not *E. coli,* but of the genus *Bacteroides* (Harmsen *et al.* 2002). Many methods have recently been designed to detect members of the *Bacteroides* genus based upon their 16S rRNA gene (Bernhard & Field 2000; Matsuki *et al.* 2002, 2004; Layton *et al.* 2006; Kildare *et al.* 2007; Okabe *et al.* 2007) and gyrB gene (Lee & Lee 2010). The use of these methods for fecal source tracking is gaining momentum and can be useful in understanding the causes of advisory conditions at Ohio's beaches.

In this study, we employed multiple assays for *Bacteroides fragilis* and *Bacteroides*-group members. We also employed a novel immunomagnetic separation method for *B. fragilis*, as well as traditional culture-based methods. The associations observed between the results of the PCR assays and other methods has the potential to contribute to a greater understanding of human fecal contamination at Lake Erie beaches, which may assist in understanding other non-human fecal contamination.

Purpose and Scope

The main purpose of this project was to address the two key issues in current beach water quality monitoring: developing and exploring new fecal indicators and rapid methods. Our primary objective was to investigate the significance of *Bacteroides* as a new fecal indicator in Lake Erie beach water as well as compare its relationship with the current fecal indicator, *E. coli*, and to develop rapid methods. The specific aims were to 1) develop rapid and robust methods for detecting *B. fragilis* from beach water using qPCR and immunomagnetic separation/ATP bioluminescence (IMS-ATP); 2) to compare the rapid methods and the conventional membrane filtration method; 3) to determine the association between the *Bacteroides*, *E. coli* and other measures of water quality parameters.

Methods

Collection of Water Samples. Water samples were collected 15 times from 2 beaches (Villa Angela and Euclid) located at Cleveland Lakefront State Park during the 2010 swimming season with support of the Ohio Division of Parks and Recreation (Table 1, Figure 1). Water samples were collected from beach waters in accordance with the Ohio Department of Health sampling guidelines for beach waters (ODH 2009). Water collection at all beaches occurred where the water was approximately three feet deep and in the same vicinity of the beach during each collection. The water was collected in sterile Whirl-pak bags by sweeping the container one foot below the water surface. Sufficient quantities of water were transported to a temporary

field lab at the OSU- Extension office in Painesville (Lake County, Ohio), which is in proximity to the sampling locations. Other water quality characteristics were collected in conjunction with a YSI water quality data sonde (Yellow Springs Instruments, Yellow Springs, Ohio) and Hach turbidimeter (Hach Company, Loveland, Colorado).

Table 1. Location of the two study beaches along the Lake Erie shore.

Beach	Latitude	Longitude	State	County	City	Zip
Villa Angela	41° 35′ 9″ N	81° 34′ 1″ W	Ohio	Cuyahoga	Cleveland	44108
Euclid	41° 35′ 2″ N	81° 34′ 9″ W	Ohio	Cuyahoga	Cleveland	44108

Figure 1. Sampling locations at Euclid and Villa Angela Beaches.

Quantifying Human Fecal Markers. Beach water samples were first pre-filtered through a filter with a larger pore size (20 µm) to remove algae and other debris that may interfere with PCR results. A total of 200 mL were pre-filtered and subsequently filtered through a 47 mm diameter membrane with a 0.45 µm pore size. Filter membranes were placed in sterile 50 mL tubes and frozen at -80°C until qPCR analysis was performed at the Ohio State University –Main Campus in Dr. Jiyoung Lee's laboratory. Upon removing filter membranes for qPCR analysis, DNA was extracted. Sonication and centrifugation were employed to detach cells and concentrate DNA efficiently. DNA was extracted using a QIAamp® DNA stool mini kit (Qiagen, Valencia, CA) with modification. DNA concentrations were determined with a NanoDrop system (Nanodrop Technologies, Wilmington, DE).

The real-time PCR assay was performed for three genetic markers associated with human fecal contamination. The following markers were amplified enabling quantification of fecal indicator density in the Lake Erie Water Samples:

1) Human-associated fecal marker, HuBac (Okabe et al. 2007)
2) Human-associated fecal marker, 16S rRNA Bacterpodes-Group (Matsuki et al. 2002)
3) Human-specific fecal marker, gyrB (Lee et al. 2010)

Immunomagnetic Separation/ ATP Bioluminescence (IMS/ATP) – *Estimation of B. fragilis.* The immunomagnetic capture uses uniform superparamagnetic polystyrene beads. The beads were coated *with B. fragilis* antibodies (Novus Biologicals, Inc.) in accordance with a procedure for coupling antibody to the magnetic beads as purchased from Bangs Laboratories, which enables the forming of a bead/bacteria complex that was easily separated from a natural heterogeneous bacterial suspension by exposure to a magnetic field. The procedure was similar to the approach used to estimate *E. coli* at freshwater beaches (Lee and Deininger, 2004; Bushon *et al.* 2009) and enterococci at marine beaches (Lee and Deininger, 2010).   The IMS/ATP method was applied to bacteria after detaching the microorganisms from the final membrane filter. With bacteria removed from the filter and placed in solution, *B. fragilis* - antibody coated beads were added and mixed for 15 min at 60 rpm with a mixer (Dynal, Lake Success, NY). During mixing, the antibody-coated beads were presumed to have bound to *B. fragilis* to form complexes. These complexes will be separated to the magnet side of the tube wall and the supernatant was discarded. Bead-bacteria complexes were resuspended in a PBS solution (10 mL) after several washing steps, the bacteria cells were lysed to free cellular DNA. This suspension containing the cellular DNA of the target organisms was placed in a Filtravette™ cuvette with luciferase being added to emit bioluminescent light. This reaction was measured in relative light units (RLUs) using a microluminometer (Model 3550, New Horizons Diagnostics, Columbia, MD).

Membrane Filtration Methods for Traditional Indicators. *E. coli* and enterococci were quantified by using modified m-TEC agar and mEI agar, respectively as done in EPA Method 1603 and EPA Method 1600. Sample volumes of 100 mL, 50 mL and 25 mL were vacuum filtered via a filtration apparatus through a 47 mm diameter membrane with a 0.45 µm pore size.   Using aseptic techniques, the filter membrane was be placed on each plate and incubated

at the appropriate temperature (*E. coli* at 35°C for 2 h, then 44.5°C for 22 h, enterococci at 41°C for 24 hours.) After incubation, magenta (*E. coli*) and blue (enterococci)-colored colonies were counted as colony forming units.

Results

Positive signals for human-associated fecal contamination were detected at both beaches using both rapid methods (quantitative PCR and IMS-ATP). Additionally, positive signals were detected using all three DNA markers indicative of human fecal contamination.

Overall, the greatest amount of fecal contamination cumulatively was at Villa Angela Beach (Figure 2). Additionally, the huBac and Bfg (*B. fragilis* 16s group-specific method) provided comparable cumulative results at both beaches (Figure 2). The gyr B method, which is believed to be more selective of human-specific fecal contamination amplified the least amount.
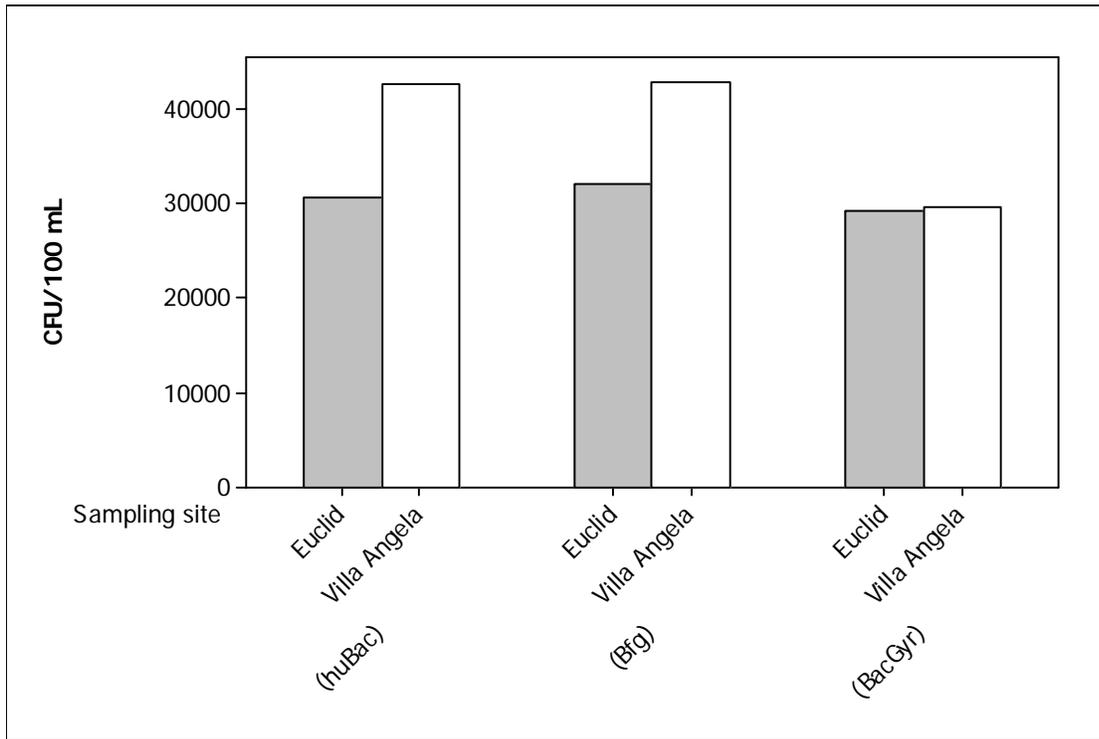

Figure 2. Sampling locations at Euclid and Villa Angela Beaches.

The relationship between the HuBac method and the group-specific method (Bfg or *B. fragilis* 16s Group-Specific method) is quite strong at the daily level as well. The two are statistically correlated (p = 0.000) and among the two, the association is quite strong for fecal indicators (R^2=0.45) as depicted in Figure 3. The relationship between HuBac and the gyr B method is not as strong (R^2 = 0. 27) , but still statistically correlated (p = 0.002) (Figure 4).

log(16s Bfg cfu/100 ml) = - 1.319 + 1.175 log(huBac cfu/100 ml)

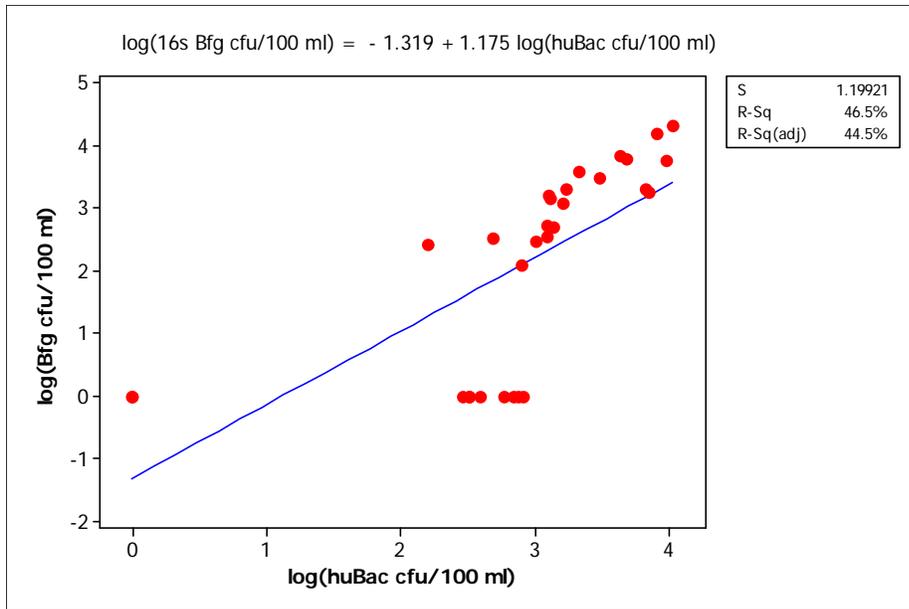| S | 1.19921 |
| R-Sq | 46.5% |
| R-Sq(adj) | 44.5% |

Figure 3. Linear regression comparing the HuBac and group-specific (Bfg) human-associated fecal genetic markers across 30 Lake Erie (Ohio) beach samples collected at Euclid and Villa Angela Beaches.
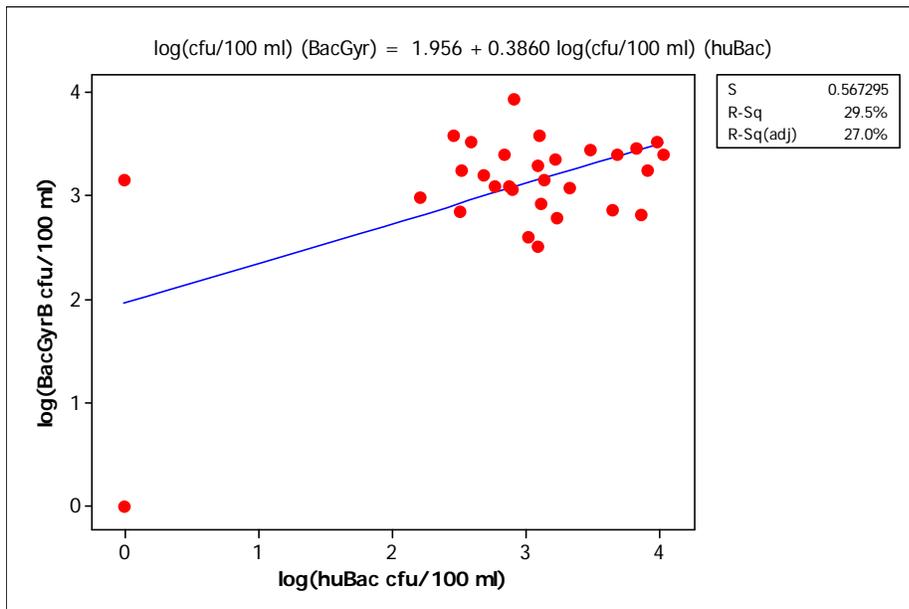

log(cfu/100 ml) (BacGyr) = 1.956 + 0.3860 log(cfu/100 ml) (huBac)

S	0.567295
R-Sq	29.5%
R-Sq(adj)	27.0%

Figure 4. Linear regression comparing the HuBac and the gyr B human-associated fecal genetic markers across 30 Lake Erie (Ohio) beach samples collected at Euclid and Villa Angela Beaches.

The IMS-ATP results suggest a slightly elevated higher amount of *B. fragilis* at Euclid Beach (Figure 5). Since the IMS-ATP method employed in this study is designed to be specific to the human-associated bacteria *B. fragilis*, it can be hypothesized that human contamination is the

predominant fecal source at this beach. In Figure 2, we see the other *B. fragilis* specific-method, gyr B qPCR, not amplifying as much DNA (and hypothetically bacteria) as the other more generic human-associated methods (huBac and B.frg (16s)) in Villa Angela Beach. This logic supports the notion that additional inputs of fecal contamination at Villa Angela Beach are likely from non-human sources (e.g. gulls, geese) at a greater level than present at Euclid Beach.
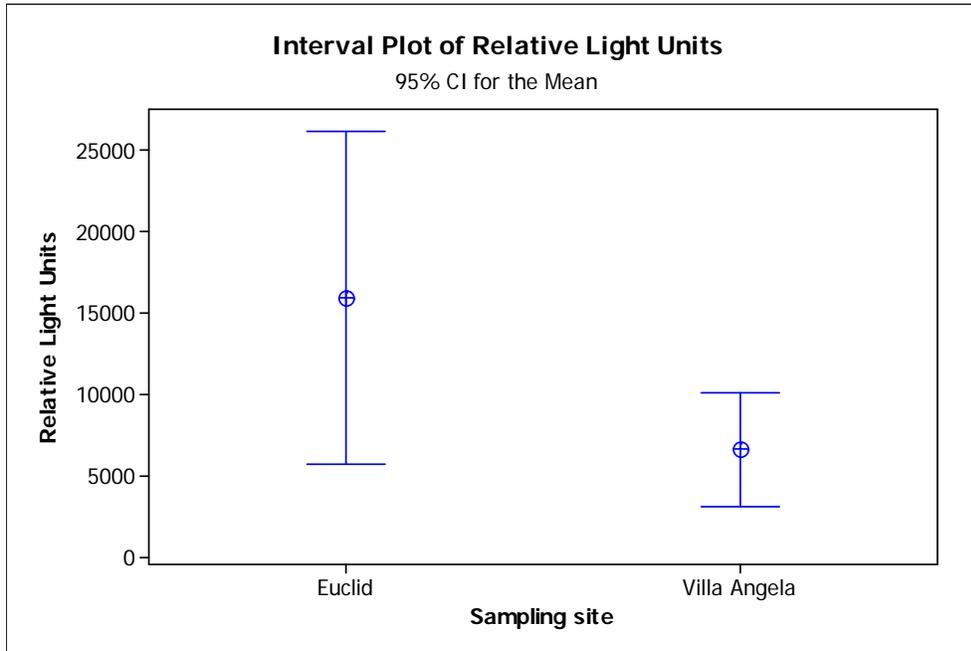

Figure 5. Interval plot comparing depicting the mean amount of relative light units (RLUs) produced by the IMS-ATP *B. fragilis* method across 30 Lake Erie (Ohio) beach samples collected at Euclid and Villa Angela Beaches.

The results definitely point to the presence of human-associated sources playing a significant role in the water quality at Euclid and Villa Angela Beaches. However, the human-associated sources as detected in this study by IMS-ATP and genetic markers are not currently able to explain the overall water quality as described by the criteria indicator *E. coli* because E. coli may come from various fecal sources as well as human source. Our results using the huBac marker do demonstrate a statistically significant Pearson correlation with the traditional culture-based *E. coli* method results (p = 0.018); however, the overall strength of the association is weak (R^2 = 0.15) as observed in Figure 6.
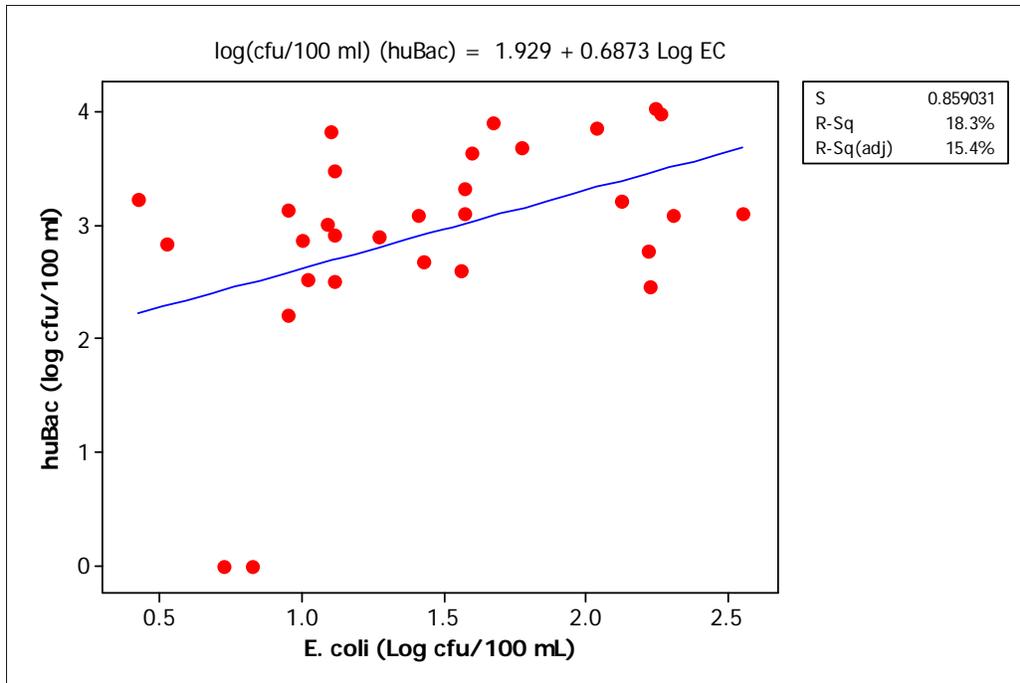
Figure 6. Linear regression comparing the huBac and log cfu/100 mL *E. coli* plate count results across 30 Lake Erie (Ohio) beach samples collected at Euclid and Villa Angela Beaches.

Conclusions and Next Steps

The data are quite clear in demonstrating a significant human-associated fecal contamination problem at these two beaches. A more thorough sanitary survey of the area may assist in narrowing the likely locations or sources of the human fecal contamination problem. However, it is apparent, that a non-human fecal contamination source (e.g. water fowls) is also likely present. Given gull and goose droppings and sightings of these species daily during the sampling period, these shorebirds/waterfowl are suspected to be also contributing to the contamination problems at these two beaches.

Because of the support of Ohio's Lake Erie Protection Fund, we have been able to gather a substantial amount of useful data that will further leverage additional financial support to more confidently understand the extent of the human/shorebird fecal contamination problem at these two beaches. We are very grateful to the Ohio Lake Erie Commission for this opportunity, as the methodology development portion of this grant enabled us to strengthen our beach monitoring capabilities of our laboratory. It was extremely valuable in assisting us with securing a US EPA Great Lakes Restoration Initiative grant that will allow us to further understand the sources and events associated with fecal contamination, particularly using shorebird fecal indicators coupled with weather phenomena.

References

Bernhard, A. E. & Field, K. G. 2000 A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. *Appl Environ Microbiol* **66**(10): 4571-4574.

Bushon, R. N., A. M. Brady, et al. 2009. Rapid detection of Escherichia coli and enterococci in recreational water using an immunomagnetic separation/adenosine triphosphate technique. *Journal of Applied Microbiology* 106(2): 432-441.

Converse, R. R., J. F. Griffith, and R.T. Noble 2009. Rapid QPCR-based assays for fecal Bacteroides and Enterococcus speciation as tools for assessing fecal contamination in recreational waters, J. Water Res. 43:4828–4837.

Dorfman, M., and K. S. Rosselot. 2009. Testing the Waters: A Guide to Water Quality at Vacation Beaches. National Resources Defense Council. http://www.nrdc.org/water/oceans/ttw/titinx.asp.

Harmsen, D., J. Rothganger, M. Frosch, and J. Albert. 2002. RIDOM: Ribosomal Differentiation of Medical Microorganisms Database. Nucleic Acids Res 30: 416-417.

Holdernan, L. V., I. J. Good, and W. E. C. Moore. 1976. Human fecal flora: variation in bacterial composition within individuals and a possible effect on emotional stress. Appl. Environ. Microbiol. 31:359-375.

Kildare, B. J., Leutenegger, C. M., McSwain, B.S., Bambic, D. G., Rajal, V. B. & Wuertz, S. 2007 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: a Bayesian approach. *Water Res* **41**(16): 3701-3715.

Layton, A., L. McKay, D. Williams, V. Garrett, R. Gentry, and G. Sayler. 2006. Development of Bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl. Environ. Microbiol. 72:4214-4224.

Lee, J. & Deininger, R. A. 2004 Detection of E-coli in beach water within 1 hour using immunomagnetic separation and ATP bioluminescence. *Luminescence* **19**(1): 31-36.

Lee, J., and R.A. Deininger. 2010. Rapid detection of enterococci in marine beach water by immunomagnetic capture and bioluminescence and its comparison with conventional methods. *Journal of Environmental Health* 72:9:8-13.

Lee, C., and J. Lee. 2010. Evaluation of new *gyrB*-based real-time PCR system for the detection of *B. fragilis* as an indicator of human-specific fecal contamination. *Journal of Microbiological Methods.* 82:311-318.

Matsuki, T., K. Watanabe, J. Fujimoto, Y. Miyamoto, T. Takada, K. Matsumoto, H. Oyaizu, and R. Tanaka. 2002. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl. Environ. Microbiol. 68:5445-5451.

Matsuki, T., Watanabe, K., Fujimoto, J., Takada, T. & Tanaka, R. 2004 Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Appl Environ Microbiol* **70**(12): 7220-7228.

Ohio Department of Health. 2009. Sampling Procedures – Beach Monitoring. Online document. http://www.odh.ohio.gov/odhPrograms/eh/bbeach/beach_procedures.aspx. Ohio Department of Health, Columbus, Ohio.

Ohio Environmental Protection Agency. 2008. Delisting targets for Ohio Areas of Concern. Columbus, Ohio. 85 pp.

Okabe, S., N. Okayama, O. Savichtcheva, and T. Ito. 2007. Quantification of host-specific Bacteroides-Prevotella 16S rRNA genetic markers for assessment of fecal pollution in freshwater. Appl Microbiol Biotechnol 74:890-901.

Simpson, J. M, J. W. Santo Domingo, and D. J. Reasoner. 2002. Microbial source tracking: state of the Science. Environ. Sci. Technol. 36:5279-5288.

U.S. Environmental Protection Agency. 2002. Method 1603: *Escherichia coli* (*E. coli*) in Water by Membrane Filtration Using Modified membrane-Thermotolerant *Escherichia coli* Agar (Modified mTEC). U.S. Environmental Protection Agency Report EPA-821-R-02-023, Washington D.C.

U.S. Environmental Protection Agency. 2007. Critical path science plan for the development of new or revised recreational water quality criteria. U.S. Environmental Protection Agency Report 823-R-08-002.